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Tezacitabine enhances the DNA-directed effects of fluoropyrimidines in human colon cancer cells and tumor xenografts

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ABSTRACT

Tezacitabine is a nucleoside analogue characterized by a dual mechanism of action. Following intracellular phosphorylation, the tezacitabine diphosphate irreversibly inhibits ribonucleotide reductase, while the tezacitabine triphosphate can be incorporated into DNA during replication or repair, resulting in DNA chain termination. In the present study we have investigated the effect of the combination of tezacitabine and 5-fluorouracil (5-FU) or 5-fluoro-2′-deoxyuridine (FUdR) on HCT 116 human colon carcinoma cells and xenografts. We used response surface analysis (RSA) and clonogenic assay to evaluate combination effects of tezacitabine and 5-FU. Tezacitabine is antagonistic when combined with 5-FU in the RSA assay and does not effect the clonogenicity of HCT 116 cells when compared with cells treated with 5-FU alone. However, when combined sequentially with FUdR, tezacitabine leads to potentiation of cell killing in the clonogenic assay, additivity in the RSA assay, and increased apoptosis when compared to FUdR alone, suggesting that cytotoxicity of fluoropyrimidines such as FUdR that have more DNA-directed effects can be potentiated by tezacitabine. We also report that oral administration of the fluoropyrimidine capecitabine, an oral prodrug of 5-FU, in combination with tezacitabine shows statistically significant additivity in the HCT 116 xenograft model. This interaction may be explained by the finding that tezacitabine elevates activity of thymidine phosphorylase (TP), the enzyme required for activation of the capecitabine prodrug in tumors. Our results provide evidence that tezacitabine enhances the DNA-directed effects of fluoropyrimidines in human colon cancer cells and may modulate the antitumor activity of fluoropyrimidines.

1. Introduction

Nucleoside analogues have proven to be effective in the treatment of a number of different types of cancer [1]. The nucleobase fluorouracil is a component of the standard of care in colorectal cancer, as is the deoxycytidine analog gemcitabine in pancreatic cancer. A number of nucleoside analogues, such as fludarabine and cladribine, have established roles in

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the treatment of hematologic malignancies. New regimens and combinations of nucleoside analogues with other anticancer agents are likely to provide increased patient benefit. The optimal use of nucleoside analogues alone and in combination requires an understanding of the preclinical mechanistic interactions between these agents [2].

In contrast to other nucleoside analogues that appear to act by inhibiting DNA replication, the deoxycytidine analogue tezacitabine [(*E*)-2′-deoxy-2′-(fluoromethylene)cytidine; FMdC] is characterized by a dual mechanism of action. Upon entering the cell, tezacitabine becomes phosphorylated by deoxycytidine kinase (dCK), forming both the di- and triphosphate analogs. The diphosphate and triphosphate analogs of tezacitabine have different activities. The diphosphate analog binds to and inhibits the enzyme ribonucleotide reductase (RNR) [3], which catalyzes the biosynthesis of deoxyribonucleosides, the rate-determining step in DNA biosynthesis. The triphosphate form of tezacitabine is incorporated into DNA during replication or repair [4]. Once incorporated into DNA, tezacitabine becomes a poor substrate for further chain elongation by DNA polymerases, resulting in DNA chain termination at the sites of incorporation. RNR inhibition by tezacitabine diphosphate results in decreased deoxynucleoside triphosphate (dNTP) pools, thus facilitating the DNA incorporation of the chain terminator tezacitabine triphosphate analog, indicating that the two activities of tezacitabine work in concert to cause cytotoxic activity. In addition, tezacitabine is relatively resistant to inactivation by the metabolic enzyme cytidine deaminase, and thus appears to be a more potent nucleoside analog than gemcitabine [5].

Tezacitabine has shown potent pro-apoptotic activity against a broad range of human hematological and solid tumor cell lines [6–8] and human tumor xenografts [9,10]. It has proved to be a radiosensitizer *in vitro* and *in vivo* as a single agent [11] or in combination with pentoxyfilline [12] or with IUdR [13]. Additionally, tezacitabine has been observed to have antiangiogenic activity [14].

Preclinical studies have suggested a potential clinical utility of tezacitabine in combination with the fluoropyrimidine 5-fluorouracil (5-FU), cisplatin, and other agents [15,16]. In clinical studies, tezacitabine administered in combination with 5-FU in a phase I dose escalation study resulted in stable disease and partial responses [17].

5-FU is widely used in a variety of therapeutic combinations for the treatment of solid malignancies [18], particularly for colorectal cancer. 5-FU antitumor activity is caused by two different mechanisms of action (Fig. 1). The first is inhibition of thymidylate synthase (TS), the enzyme responsible for the reductive methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). The active metabolite of 5-FU, 5′-FdUMP forms a covalent ternary complex with TS and the reduced folate cofactor, 5,10-methylenetetrahydrofolate, to inhibit TS, resulting in a thymine-less state, DNA damage, and apoptosis [19]. A second mechanism of 5-FU-induced cytotoxicity is due to the metabolism of the drug to ribonucleotides and incorporation of FUTP into RNA and altered processing of RNA species [20].

Tezacitabine, like 5-FU, contributes to the depletion of dTTP pools by its potent inhibition of RNR, although antitumor activity can also be explained by incorporation

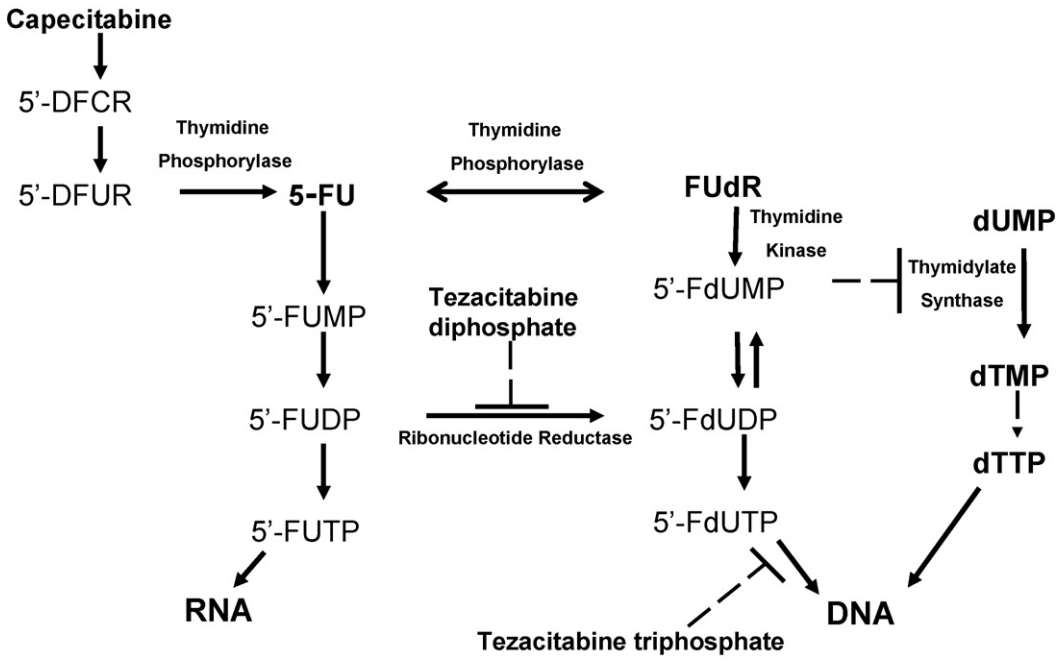


Fig. 1 – Metabolism and actions of fluoropyrimidine drugs 5-fluorouracil (5-FU), 5-fluoro-2′-deoxyuridine (FUdR) and N4-pentyloxycarbonyl-5′-deoxy-5-fluorocytidine (capecitabine) and their potential interactions with tezacitabine diphosphate (FMdCDP) and triphosphate (FMdCTP) analogs. Abbreviations: 5′-DFCR = 5′-deoxy-5-fluorocytidine; 5′-DFUR = 5′-deoxy-5-fluorouridine; 5′-FUMP, 5′-FUDP, 5′-FUTP = 5-fluorouridine-5′-mono-, di-, tri-phosphate; 5′-FdUMP, 5′-FdUDP and 5′-FdUTP = 5-fluoro-2′-deoxyuridine-5′-mono-, di-, tri-phosphate; dTTP = deoxythymidine triphosphate. Dashed lines indicate inhibition of the relevant enzymatic reaction.

of its triphosphate metabolite into a growing DNA strand. In addition, tezacitabine-induced RNR inhibition depletes endogenous pools of deoxyuridine monophosphate (dUMP), resulting in enhanced binding of 5′-FdUMP to TS. The mechanisms of action of these two drugs, and the preclinical and clinical data described above, suggest that the combination of tezacitabine and 5-FU may lead to an enhanced dNTP pool perturbation and hence greater antitumor activity compared to either alone. This rationale prompted us to examine whether the combination would enhance cytotoxicity. We investigated the responses of HCT 116 human colon carcinoma cells to tezacitabine alone or in combination with 5-FU *in vitro* and *in vivo* as subcutaneous tumors in athymic mice. We characterized the effects of sequential or simultaneous exposure to tezacitabine and 5-FU on cell survival and proliferation. Since we did not observe a synergistic interaction between the two drugs in either cell-based assays or in a xenograft model, we investigated whether tezacitabine affected the TS inhibitory activity of 5-FU by directly assessing TS enzymatic activity in HCT 116 cells. In addition, we evaluated the effects of tezacitabine and 5-fluoro-2′-deoxyuridine (FUdR), a more selective inhibitor of TS than 5-FU, combined with a sequential or simultaneous treatment schedule. We report here that sequential treatment with tezacitabine followed by FUdR is much more effective in inhibiting survival and proliferation of HCT 116 cells than FUdR alone and that this effect results in increased apoptosis and DNA damage. Finally, we evaluated the combination of tezacitabine and capecitabine (N4-pentyloxycarbonyl-5′-deoxy-5-fluorocytidine, Xeloda®), an orally administered fluoropyrimidine that is converted to 5-FU by the enzyme thymidine phosphorylase (TP) [21], in an HCT 116 xenograft model. Our findings show that this combination produces a statistically significant additivity. This interaction may be correlated with a tezacitabine-induced elevation of TP levels in HCT 116 tumor tissue, leading to an increased conversion of capecitabine to 5-FU.

2. Materials and methods

2.1. Drugs

Tezacitabine [(*E*)-2′-deoxy-2′-(fluoromethylene)cytidine; FMdC] was manufactured by Chiron Corp. and supplied in glass vials containing 100 mg sterile lyophilized powder. Drug was reconstituted with 10 mL of 0.9% sodium chloride for injection (saline), to give a 39 mM (10 mg/mL) stock solution. Aliquots of the stock solution were immediately frozen at −20 °C; each aliquot was thawed only once and further diluted in tissue culture medium immediately before each experiment. 5-FU was purchased from ICN Pharmaceuticals (Costa Mesa, CA) as 10 mL vials containing 500 mg 5-FU in aqueous solution (384.4 mM). FUdR was purchased from Sigma–Aldrich (St. Louis, MO) and dissolved in sterile water as a 47 mM solution (11.6 mg/mL). Capecitabine was purchased from Roche Pharmaceuticals (Nutley, NJ) as film coated tablets for oral administration and formulated for *in vivo* studies in 40 mM citrate and 5% gum Arabic at 21.88 mg/mL.

2.2. Cell survival (clonogenic survival assay)

HCT 116 human colon carcinoma cells were obtained from ATCC (Manassas, VA) and maintained in RPMI-1640 growth medium supplemented with 10% fetal bovine serum and 1% glutamine. Cells were plated in 6-well plates, and, after 24 h, were treated simultaneously with tezacitabine and 5-FU/FUdR or with each single drug for 24 h. At the end of each treatment time, cells were trypsinized, counted, and replated (range 50–2000 cells/well) in drug-free medium for 10–15 days. Resulting colonies (>50 cells) were scored after staining with crystal violet.

For combination experiments, tezacitabine was used at the fixed concentration of 20 nM and combined in different schedules with multiple concentrations of 5-FU or FUdR. Sequential combinations were accomplished with an initial exposure to either tezacitabine or to 5-FU or FUdR for 24 h; after 24 h the first drug was removed, cells were washed with PBS, and the second drug was added.

2.3. Response surface analysis (RSA)

HCT 116 cells were plated into black and clear 384-well dishes (Greiner, Longwood, FL). Cells were exposed to a metric of combinations of 10 tezacitabine concentrations and 8 test drug concentrations (5-FU or FUdR) for a total of 80 concentration combinations in each experiment. The tezacitabine concentrations consisted of a 1.3-fold dilution series from 2×10^{-7} to 1.9×10^{-8} M. The 5-FU concentrations consisted of a 2-fold dilution series from 3×10^{-5} to 2.3×10^{-7} M. The FUdR concentrations consisted of a 2.3-fold dilution series from 2.5×10^{-5} to 3.1×10^{-8} M. The EC_{50} values for these drugs are approximately 7×10^{-8} M, 3×10^{-6} M, and 8×10^{-7} M for tezacitabine, 5-FU, and FUdR, respectively. Each drug was run in combination with itself as a control. The plate design consisted of one quadrant with the tezacitabine dilution series + vehicle, one quadrant with the 5-FU or FUdR dilution series + vehicle, one quadrant with the drug combination matrix of tezacitabine + 5-FU or FUdR, and one quadrant containing vehicle only (untreated controls). Each drug pair was given on three different schedules: (i) simultaneous addition of both drugs 24 h after plating; (ii) addition of tezacitabine 24 h after plating and 5-FU/FUdR 48 h after plating; (iii) addition of 5-FU/FUdR 24 h after plating and tezacitabine 48 h after plating. In the tezacitabine plus 5-FU experiments, tezacitabine was washed out with PBS after a 24-h exposure and fresh media applied to the cells, while 5-FU exposure was continuous, to mimic the bolus administration of tezacitabine and continuous infusion of 5-FU used clinically [17]. In the tezacitabine plus FUdR experiments, the drugs were washed out after 24 h of exposure. Viable cell number was determined 120 h after plating using Cell Titer Glo (Promega, Madison, WI) and reading luminescence intensity with a Victor luminometer (Perkin-Elmer Wallac, Wellesley, MA). Cell plating, drug addition, PBS washes, and the addition of Cell Titer Glo were performed with an FX robot (Beckman-Coulter, Fullerton, CA).

The data were analyzed by plotting the deviation from additivity: the difference between the observed inhibition

effect and the effect predicted by the Loewe additivity model [22]:

$$\Delta = E_{obs} - E_{add}$$

where Δ is the deviation from additivity, E_{obs} the observed cell number normalized to the untreated controls, and E_{add} is the additive effect predicted by the Loewe additivity model. The parameters for the additive model, the EC_{50} and Hill slope for each drug, were determined from the two quadrants treated with each drug alone. A key feature of Loewe's model is that a drug is additive in combination with itself, hence providing a well defined additivity control. Interaction (antagonism or synergy) was determined by observing a response surface deviation from additivity larger than the same drug combination (additive) controls, typically >10% deviation.

2.4. Thymidylate synthase activity

TS activity was measured as release of tritium from [5-^{3}H]-dUMP during the TS catalyzed reaction in an intact cell assay modified from the original methodology [23]. HCT 116 colon carcinoma cells were plated at 1×10^4 cells/well into 96-well plates (Corning Costar, Acton, MA) in 95 μL of RPMI 1640 media containing 10% fetal bovine serum and glutamine. After overnight incubation at 37 °C, cells were treated with 5 μL/well of either 20× tezacitabine, 5-FU, or FUdR diluted in cell growth media. Sequential combinations were accomplished as described for the clonogenic survival assay. Cells were incubated for 2 h at 37 °C with 10 μL/well of 1 μM ^{3}H-deoxycytidine (specific activity 22.0 Ci/mmol, GE Healthcare, Piscataway, NJ). The cell supernatant was removed from the cells and put into a new 96-well plate. The cell monolayer, after removal of the cell supernatant, was treated with 100 μL/well of Cell Titer Glo assay reagent (Promega, Madison, WI) and luminescence from the plates was then read on a Beckman Coulter Affinity machine. An equal volume of 6% activated charcoal powder (Mallinckrodt, Hazelwood, MO) in water was added to and mixed with the supernatant. The cell supernatant and activated charcoal were incubated together at room temperature for at least 15 min. The activated charcoal containing the free tritium was spun down through the cell supernatant mixture and pelleted at the bottom of the well by spinning the plates for 5 min at 2000 rpm at room temperature. Thirty microlitres of the clear charcoal supernatant containing tritiated water was collected from each well and put into the wells of 96-well plates (Perkin-Elmer Wallac, Wellesley, MA) containing 150 μL/well of OptiPhase "SuperMix" scintillation fluid (Perkin-Elmer Wallac, Wellesley, MA). Data was recorded as CPM for each well.

The TS assay data were normalized for cell number and viability. Experimental values were calculated using the following formulas:

$$\%\,\text{Inhibition of TS activity} = 100 - \left(\left[\frac{\text{CPM treated sample}}{\text{CPM media control}}\right] \times 100\right)$$

$$\%\,\frac{\text{Inhibition for cell number}}{\text{viability using Cell Titer Glo(CTG)}} = 100 - \left(\left[\frac{\text{CTG units treated sample}}{\text{CTG units media control}}\right] \times 100\right)$$

2.5. Induction of apoptosis by tezacitabine and 5-FU/FUdR

HCT 116 human colon carcinoma cells were treated with tezacitabine for 3, 6, 9, 24, or 48 h, and induction of apoptosis was measured at each time point as activation of caspases using the Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega, Madison, WI) according to the manufacturer's protocol. The same assay was also used to investigate Caspase-3/7 activation after treatment of HCT 116 cells with 20 nM tezacitabine in sequential or simultaneous combination with 5-FU/FUdR. In addition, cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) were detected by the Cell Death Detection ELISA kit (Roche, Indianapolis, IN) in supernatants of HCT 116 cultures at the end of 24-h treatment with 20 nM tezacitabine in sequential or simultaneous combination with 5-FU/FUdR.

2.6. Induction and repair of DNA damage after treatment with tezacitabine and FUdR

The induction and repair of DNA damage in HCT 116 cells after treatment with tezacitabine alone (20 nM × 24 h) or in combination with FUdR (1.2 μM) for 24 h was determined by alkaline single-cell gel electrophoresis, also known as the comet assay [24]. This assay measures increased DNA migration as a sign of the presence of DNA strand breaks, alkali labile sites, and DNA excision repair sites. The assay was performed according to the manufacturer's protocol (Trevigen, Gaithersburg, MD). The percentages of DNA in the comet head and comet tail were measured by fluorescence intensity using CASP (Comet Assay Software Project) software (http://casp.sourceforge.net/).

2.7. Thymidine phosphorylase activity

TP activity in HCT116 tumor lysates was determined by ELISA (Roche, Indianapolis, IN) with monoclonal antibodies specific to human TP and expressed in units (TP unit = TP level of standard enzyme solution which phosphorolyzes 5′-dFUrd to 5-FU at a rate of 1 μg of 5-FU/h).

2.8. HCT 116 xenograft studies

Animals were cared for according to NIH guidelines in an AAALAC-accredited facility. Seven- to nine-week-old female nude mice (Charles River, Hollister, CA) were subcutaneously implanted with 5×10^6 HCT 116 cells. Animals were randomized into groups of 10 animals and dosing initiated when tumor volumes reached ~200 mm^3. Tezacitabine (100 mg/kg) or vehicle was given by IP injection, while capecitabine (175 mg/kg) was given orally for 14 days. The drug combinations were administered on three different schedules: a single injection of tezacitabine given on day 1, with capecitabine administered on days 1–14 (simultaneous treatment); tezacitabine given on day 1, followed by capecitabine on days 2–15; or capecitabine treatment initiated on day 1, with a single dose of tezacitabine given on day 2. In the combination groups treated on the same day, tezacitabine was administered first, followed by capecitabine 30 min later. Tumor volumes and weights were measured twice weekly, and animals were monitored for clinical signs. Partial regressions (PRs) were

defined as a 50–99% decrease from initial tumor size, and complete regressions (CRs) as a 100% decrease.

A repeat measures interaction model [25] was used to determine the significance of drug (capecitabine) × drug (tezacitabine) × time interaction. Potentiation of tumor growth inhibition was defined as any deviation of the model from additivity, as indicated by a statistically significant interaction term in a least squares linear model. Least squares analysis of tumor volumes was performed with the SAS PROC-GLM software package.

3. Results

3.1. *Cytotoxic and antiproliferative response of human cancer cells to tezacitabine and fluoropyrimidine combinations*

For clonogenic experiments we selected a concentration of tezacitabine (20 nM) that did not induce cytotoxicity of HCT 116 colon cancer cells after a 24-h exposure. Cells were treated with the following regimens: (1) simultaneous exposure to tezacitabine and 5-FU; (2) 5-FU first for 24 h, a wash out, followed by tezacitabine for a further 24 h; (3) tezacitabine first for 24 h, a wash out, followed by 5-FU for a further 24 h. In all cases, tezacitabine did not enhance the activity of 5-FU on the cells (Fig. 2, panel A).

We also analyzed the effects due to this drug combination using the RSA assay. This assay [22] determines the additivity or degree of interaction between two drugs via exposure of cells to a combinatorial matrix of drug concentrations. Cells were plated in 384 well plates and treated with the drug combinations either simultaneously or sequentially. Each drug was also run in a matrix with itself as a control for additivity. A 10 pt dilution series of tezacitabine from 2×10^7 to 1.9×10^8 M and 8 pt dilutions of 5-FU or FUdR from 3×10^5 to 2.3×10^7 M and 2.5×10^5 to 3.1×10^8 M, respectively, were run. Ninety-six hours after the initial drug exposure, viable cells were evaluated and the deviation from additivity was calculated, in order to determine additivity or the degree of drug interaction. Additivity controls containing a matrix of tezacitabine plus tezacitabine or 5-FU plus 5-FU resulted in additivity as expected (data not shown). However, the two drugs given simultaneously for 24 h (Fig. 2, panel B) or sequentially for 24 h (Fig. 2, panel C) both showed an antagonistic interaction at 96 h. These results are consistent with the clonogenic survival studies.

To better understand the mechanistic relationship between tezacitabine and 5-FU, we evaluated how tezacitabine could modulate the effects of FUdR, which produces more

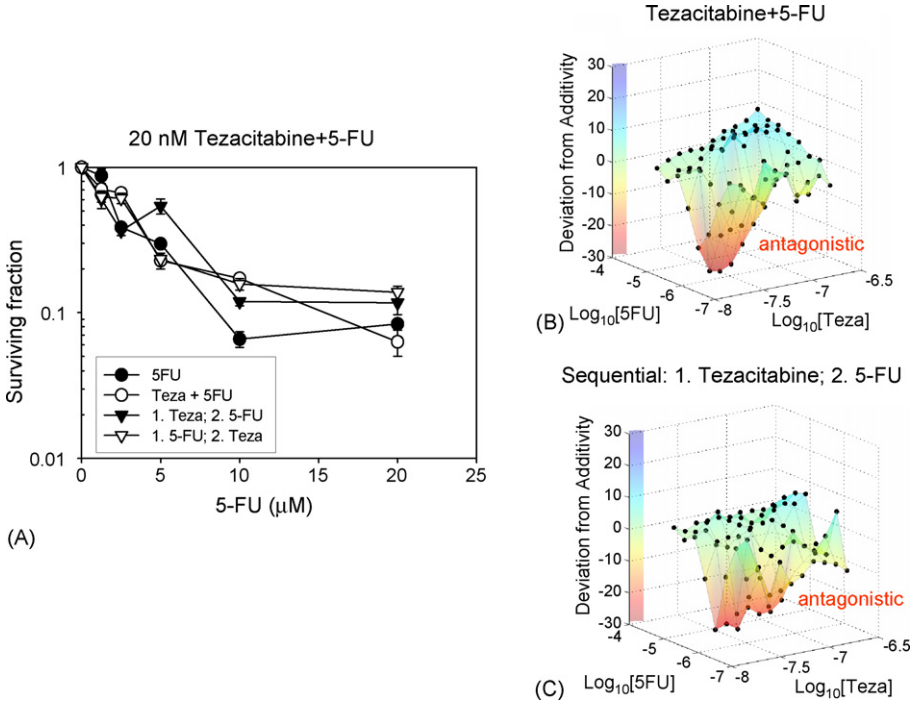


Fig. 2 – Tezacitabine does not potentiate cytotoxicity induced by 5-FU. Panel A. Clonogenic survival assay was performed as described in Section 2. HCT 116 cells were exposed to the indicated concentrations of 5-FU alone or in combination with tezacitabine. Treatments: (●) 5-FU for 24 h; (○) 20 nM tezacitabine + 5-FU simultaneous for 24 h; (▼) sequential 20 nM tezacitabine for 24 h + 5-FU for additional 24 h; (▽) sequential 5-FU for 24 h + 20 nM tezacitabine for additional 24 h. Panel B. Response surface analysis (RSA) was performed as described in Section 2. Tezacitabine and 5-FU were simultaneously added to the cells for 24 h, and washed out with PBS, with 5-FU added back to the culture media. Viable cells were measured 96 h after drug addition. Panel C. Sequential addition of tezacitabine and 5-FU also shows antagonism as assayed by RSA. Tezacitabine was applied to the cells for 24 h, washed out with PBS, and media containing 5-FU applied. Viable cells were measured 96 h after the first drug addition. In panels B and C, the *x* and *y* axes represent the drug concentrations, while the *z*-axis indicates the deviation from additivity.

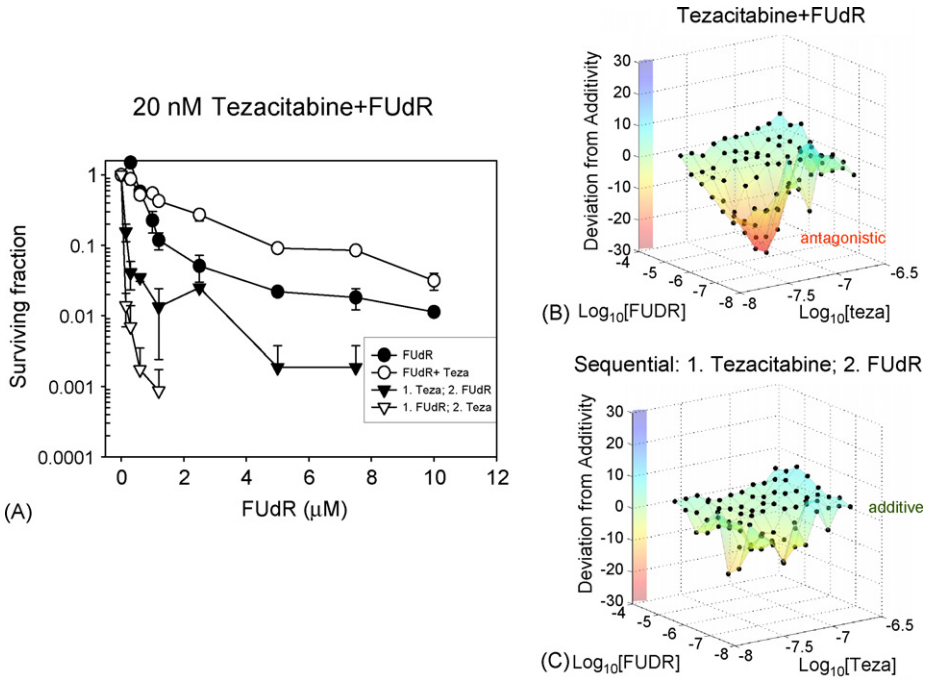


Fig. 3 – Tezacitabine enhances cytotoxicity induced by FUdR. Panel A. Clonogenic survival assay was performed as described in Section 2. HCT 116 cells were exposed to the indicated concentrations of FUdR alone or in combination with tezacitabine. Treatments: (●) FUdR for 24 h; (○) 20 nM tezacitabine + FUdR simultaneous for 24 h; (▼) sequential 20 nM tezacitabine for 24 h + FUdR for additional 24 h; (▽) sequential FUdR for 24 h + 20 nM tezacitabine for additional 24 h. Panel B. Response surface analysis (RSA) was performed as described in Section 2. Tezacitabine and FUdR were simultaneously added to the cells for 24 h, and then washed out with PBS. Viable cells were measured 96 h after drug addition. Panel C. Sequential addition of tezacitabine and FUdR shows additivity as assayed by RSA. Tezacitabine was applied to the cells for 24 h, washed out with PBS, FUdR containing media was applied for 24 h, then cells washed again and the media replaced. Plates were evaluated 96 h after the first drug addition. In panels B and C, the *x* and *y* axes represent drug concentrations, while the *z*-axis indicates the deviation from additivity.

DNA-directed cytotoxic effects than 5-FU itself. As shown in Fig. 3, panel A, in the clonogenic assay, the sequential exposure to tezacitabine followed by FUdR, or vice versa, greatly increased cytotoxicity in HCT 116 cells compared to FUdR alone. However, the simultaneous exposure of HCT 116 cells to tezacitabine plus FUdR decreased the cytotoxicity induced by FUdR alone. The RSA assay was used to probe the relationship between FUdR and tezacitabine in HCT 116 cells. As predicted, each drug in a matrix with itself resulted in an additive surface (data not shown). Similar to the case of tezacitabine and 5-FU, simultaneous treatment of the cells for 24 h resulted in antagonism at 96 h (Fig. 3, panel B). In contrast, the drugs applied sequentially (in either order) for 24 h and assessed at 96 h after the first drug addition resulted in additivity (Fig. 3, panel C). As in the tezacitabine and 5-FU studies, these results mirror those of the clonogenic survival assay.

3.2. *Biochemical effects of tezacitabine and fluoropyrimidines*

Cytotoxicity induced by fluoropyrimidines is mainly mediated by the inhibition of the enzyme TS. To characterize whether tezacitabine could inhibit TS, we measured TS catalytic activity in HCT 116 cells after 24 h exposure to tezacitabine. Fig. 4, panel A, shows that exposure to tezacitabine did not result in inhibition of TS at any of the drug concentrations tested. Given the ability of tezacitabine to potentiate the cytotoxic and antiproliferative effects of FUdR, we investigated the effects of their combination on TS inhibition. We found that sequential incubation of HCT 116 cells with FUdR followed by tezacitabine (Fig. 4, panel B) significantly increased TS inhibition compared to the effects observed after FUdR alone. A significant inhibition of TS was still evident when HCT 116 cells were exposed to 0.5 or to 0.08 μM tezacitabine for 24 h after 24-h exposure to a range of FUdR concentrations, while no TS inhibition was evident when 24-h exposure to FUdR alone was followed by 24-h incubation in drug-free medium.

3.3. *DNA damage and apoptosis*

The extent of DNA damage induced in HCT 116 cells by treatment with FUdR or tezacitabine alone, and by the two drugs in sequential or simultaneous combination, was assessed by the comet assay. Fig. 5 shows representative images of HCT 116 cells after exposure to the indicated drugs. 24-h exposure to FUdR alone or tezacitabine alone did not produce evident migration of DNA, indicative of substantial DNA integrity. An apparent diffuse DNA "tail" indicative of DNA degradation became evident in HCT 116 cells treated with

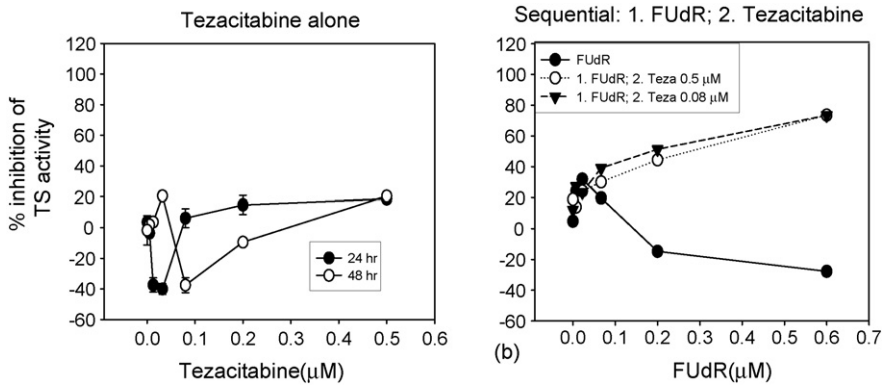


Fig. 4 – FUdR-induced thymidylate synthase inhibition is modulated by tezacitabine. Evaluation of TS inhibition was performed as described in Section 2. HCT 116 cells were exposed to the indicated concentrations of tezacitabine (panel A) or FUdR (panel B) alone or in combination with tezacitabine. Data are expressed as percentage of inhibition of thymidylate synthase activity after the indicated treatments compared to untreated control. Panel A. HCT 116 cells were exposed to tezacitabine for 24 h. TS inhibition was evaluated at the end of the treatment (●) or after additional 24 h incubation in drug-free medium (○). Panel B. TS inhibition was evaluated at the end of these treatments: (●) 24 h incubation with FUdR + additional 24 h incubation in drug-free medium; sequential 24 h incubation with FUdR + additional 24 h incubation with 500 nM (○), 80 nM (▼) or 13 nM (▽) tezacitabine.

sequential or simultaneous combinations of tezacitabine and FUdR for 24 h. Although the percentage of DNA present in tails was similar between the three treatment groups, the percentage of cells characterized by a measurable diffusion of DNA was higher in the sequential treatment groups (17% in tezacitabine followed by FUdR and 14% in FUdR followed by tezacitabine groups) than in cells exposed simultaneously to tezacitabine plus FUdR (8%).

The diffuse shape of the ''comets'' observed after cytotoxic treatments may be indicative of apoptotic cells or cells with highly damaged DNA. To understand the effects of drug exposure, we investigated whether tezacitabine was able to modulate the FUdR-mediated apoptotic response in HCT 116 cells. We first evaluated by ELISA whether and how the drug treatment induced the formation of cytoplasmic histone-associated DNA fragments in HCT 116 cells. As shown in Fig. 6, 24-h exposure to 20 nM tezacitabine did not increase the number of DNA fragments when compared to the untreated control. HCT 116 cells exposed to FUdR for 24 h presented a time-dependent increase in DNA fragments. Sequential treatment of HCT 116 cells with tezacitabine followed by FUdR, or vice versa, produced an even greater time-dependent increase in DNA fragments in both sequences tested. The simultaneous exposure of HCT 116 cells to tezacitabine plus FUdR did not significantly increase the number of DNA fragments when compared with cells exposed to FUdR alone, and this effect was not time-dependent.

We also evaluated the pro-apoptotic effects of treating HCT 116 cells with tezacitabine in combination with 5-FU or FUdR by measuring the activities of caspase-3 and -7. Fig. 7 shows that there was a moderate (two-fold) increase in caspase activity after 24-h treatment with 5-FU, but this effect was not significantly modulated by the addition of tezacitabine. When 20 nM tezacitabine was combined with increasing concentrations of FUdR in a sequential fashion, the increase in caspase activity was greatly enhanced, while the simultaneous treatment with tezacitabine plus FUdR had no effect. The pro-apoptotic effects of sequential tezacitabine and FUdR are consistent with the increased cytotoxicity observed in HCT 116 cells exposed to this drug combination.

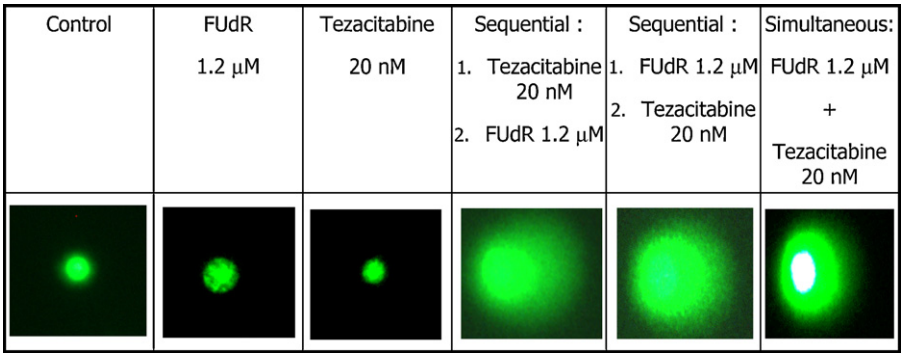


Fig. 5 – SCGE (comet assay) of HCT 116 cells after treatment for 24 h with 1.2 μM FUdR alone, 20 nM tezacitabine alone, and the sequential or simultaneous combinations of the two agents. Microphotographs are representative samples of slides containing 600 cells. Cells were processed as described in Section 2.

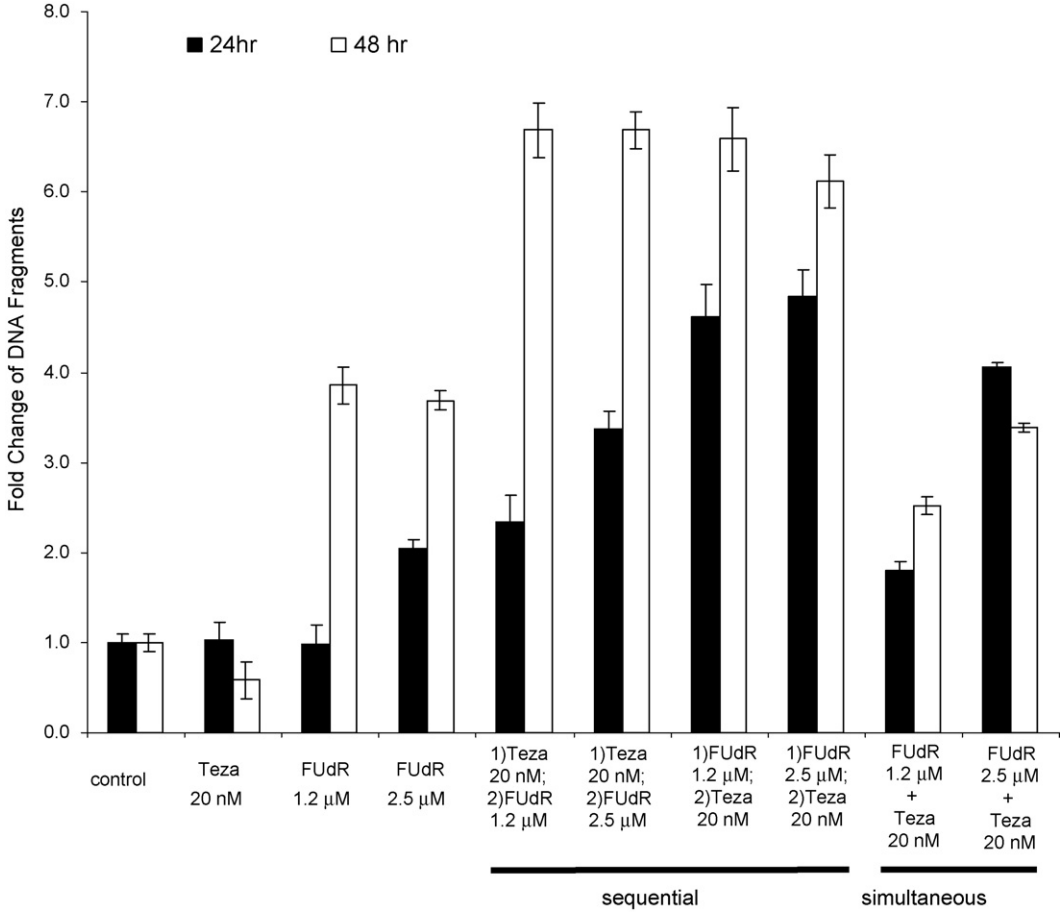


Fig. 6 – Increased number of histone-associated DNA fragments released by HCT 116 cells following sequential tezacitabine and FUdR. HCT 116 cells were exposed for 24 h to 1.2 or 2.5 μM FUdR alone, 20 nM tezacitabine alone and the sequential or simultaneous combinations of the two agents. Cell supernatants were processed at the end of the treatment (black bars) and after 24 h incubation in drug-free medium (white bars) as indicated by the manufacturer of the assay. Data are expressed as fold increase of caspase 3/7 signal over the untreated control cells.

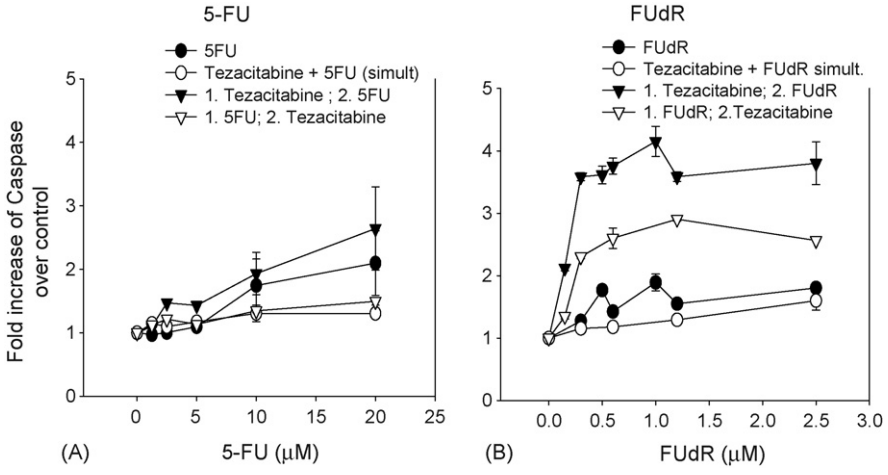


Fig. 7 – Increase in HCT 116 cell caspase 3/7 activation after sequential treatment with tezacitabine and FUdR. Panel A: (●) 5-FU for 24 h; (○) 20 nM tezacitabine + 5-FU simultaneous for 24 h; (▼) sequential 20 nM tezacitabine for 24 h + 5-FU for additional 24 h; (▽) sequential 5-FU for 24 h + 20 nM tezacitabine for additional 24 h. Panel B: (●) FUdR for 24 h; (○) 20 nM tezacitabine + FUdR simultaneous for 24 h; (▼) sequential 20 nM tezacitabine for 24 h + FUdR for additional 24 h; (▽) sequential FUdR for 24 h + 20 nM tezacitabine for additional 24 h. Cells were harvested at the end of treatment and processed as indicated by the manufacturer of the assay. Data are expressed as fold increase of caspase 3/7 signal over the untreated control cells.

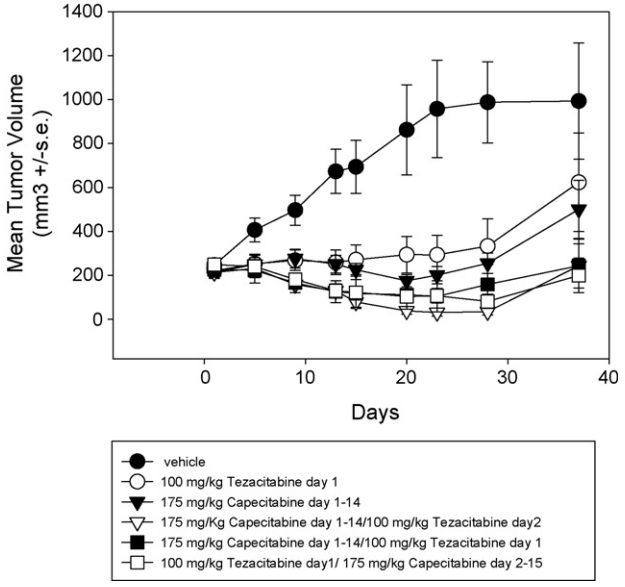
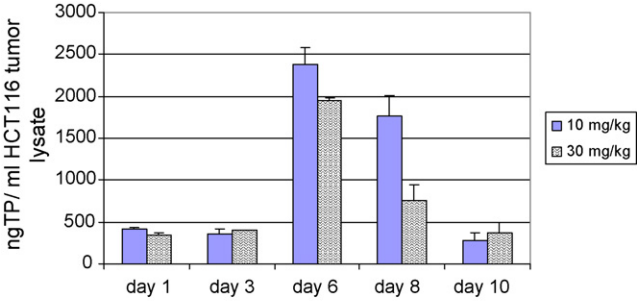


Fig. 8 – Tezacitabine and capecitabine combination in the HCT 116 xenograft tumor model shows statistically significant additivity. The experiment was performed as described in Section 2. Symbols: (●) vehicle; (○) 100 mg/kg tezacitabine on day 1; (▼) 175 mg/kg capecitabine on days 1–14; (▽) 175 mg/kg capecitabine on days 1–14 + 100 mg/kg tezacitabine on day 2; (■) 175 mg/kg capecitabine on days 1–14 + 100 mg/kg tezacitabine on day 1; (□) 100 mg/kg tezacitabine on day 1 + 175 mg/kg capecitabine on days 2–15. Data are expressed as the average tumor volume (n = 10) ± standard error.



Tezacitabine IP dose	TP (ng/ml ± SE) Day 1	TP (ng/ml ± SE) Day 6	TP (ng/ml ± SE) Day 10
10 mg/kg	422.9 ± 16.0	2383.4 ± 204.1	274.6 ± 89.9
30 mg/kg	337.9 ± 32.1	1954.7 ± 30.7	366.8 ± 123.6

Fig. 9 – Thymidine phosphorylase (TP) levels in HCT 116 xenograft tumors are increased at approximately 6 days by a single dose of 10 or 30 mg/kg tezacitabine. HCT 116 tumor xenografts were harvested at the indicated times after a single IP 10 mg/kg or 30 mg/kg dose of tezacitabine. Tumor tissues were processed according to the manufacturer instructions and TP levels were evaluated by an ELISA assay specific for human TP. Data are expressed as ng of TP/mL of tissue extract ± standard error.

3.4. *Antitumor activity of tezacitabine and fluoropyrimidines on HCT 116 tumor xenografts*

The observation in cellular assays that the interaction between tezacitabine and fluoropyrimidines was sequence-dependent led us to investigate the antitumor activity of these combination treatments *in vivo* using capecitabine, the oral fluoropyrimidine carbamate. Animals implanted subcutaneously with HCT 116 xenografts were treated with capecitabine and tezacitabine on a variety of different treatment schedules as described in Section 2 (Fig. 8). Statistical analysis showed that the drugs in combination were additive on all schedules, with a positive interaction term in a least squares linear model, indicating possible synergism. As shown in Fig. 8, each combination group exhibited tumor regressions. In the tezacitabine single agent treatment group, 3 complete responses (CR) and 1 partial response (PR) were observed. In the capecitabine alone group there were 2 CRs. The sequential treatment with tezacitabine on day 1 followed by capecitabine on days 2–15 produced 2 CRs and 2 PRs, and the opposite sequential treatment (capecitabine on days 1–14/tezacitabine on day 2) group resulted in 5 CRs and 0 PRs. Finally, in the simultaneous treatment group, 2 CRs and 1 PR were observed. Minor to severe weight loss was associated with dosing in all groups. The study was reproduced with similar results.

Capecitabine is converted to 5-FU by TP, an enzyme with high activity in many types of tumors [21] and essential for the efficacy of capecitabine and its intermediate 5′-dFUrd in human cancer xenografts [26]. Because of this effect, we investigated whether treatment of HCT 116 xenografts with tezacitabine produced an increased level of TP in xenograft tumor tissues. Fig. 9 shows TP levels in HCT 116 tumor extracts prepared at different time points following a single 10 or 30 mg/kg tezacitabine dose. We observed a significant increase in tumor TP levels 6 days after tezacitabine administration. The increased TP level of HCT 116 xenografts disappeared by day 10, at which time TP levels were comparable to those measured at day 1 of the experiment. These findings suggest that the high antitumor efficacy observed after treatment with tezacitabine plus capecitabine may result from a tezacitabine-mediated ~6-fold increase in expression of TP in HCT 116 xenografts leading to more efficient conversion of capecitabine to its active form 5-FU in the tumor cells.

4. Discussion

Nucleoside analogs are recognized as a major class of chemotherapeutic agents with antitumor activity. Cytidine nucleoside analogs such as ara-C have demonstrated activity in hematological malignancies [27], and gemcitabine is

successfully used to treat a variety of solid tumors [28]. In addition to pyrimidine nucleoside analogs, a family of purine nucleoside analogs, including fludarabine, cladribine and pentostatin, has also demonstrated significant activity, mainly in the therapy of hematological malignancies [29,30]. Cytotoxicity mediated by nucleoside analogs is generally explained by incorporation into DNA and by a DNA chain termination effect of differing potency among the analogs. In contrast with the other nucleoside analogs, tezacitabine is a cytidine analog that causes DNA synthesis perturbation by two different mechanisms of action. First, it promotes DNA chain termination following incorporation of its triphosphate metabolite, and secondly, it alters the cellular dNTP pool as a consequence of RNR inhibition by its diphosphate metabolite [4,5]. Tezacitabine also has the advantage of being less sensitive to deamination by cytidine deaminase [5] and therefore may be more active in malignancies expressing high levels of cytidine deaminase [31].

RNR inhibition induced by tezacitabine and TS inhibition induced by fluoropyrimidines like 5-FU may lead to a significant dNTP pool perturbation and increased antitumor activity compared to either drug alone. The combination of tezacitabine and 5-FU in the treatment of patients with advanced solid tumors was relatively well tolerated in a recent phase I clinical trial, and had clinical activity in patients with esophageal and other gastrointestinal cancers [32]. The aim of our study was to investigate in human colon cancer cells the mechanism of interaction between these two drugs. Our results with clonogenic and RSA assays show that no combination of these two drugs is effective in increasing cytotoxicity, and combinations actually antagonize the effects of each single drug in HCT 116 cells. The lack of potentiation of 5-FU cytotoxicity by tezacitabine observed in our studies might be explained by a predominant effect of 5-FU on RNA biosynthesis in HCT 116 cells [33]. To investigate this hypothesis, we used FUdR in place of 5-FU to maximize inhibition of TS and DNA-directed cytotoxic effects. We observed that a simultaneous exposure to tezacitabine and FUdR decreased the extent of cytotoxicity induced by FUdR alone. However sequential treatment with tezacitabine and FUdR produced a significant increase in cytotoxicity. Consistent with the increased cytotoxicity, we found that a sublethal concentration of tezacitabine was able to increase the apoptotic response in HCT 116 cells sequentially exposed to tezacitabine and FUdR.

To understand the mechanistic implications of these results, we investigated whether treatment with tezacitabine could affect the inhibition of TS mediated by 5-FU or FUdR. Interestingly, we found that FUdR-mediated TS inhibition was still high when HCT 116 cells were exposed to tezacitabine for 24 h after FUdR. One explanation for this might be that tezacitabine-mediated RNR inhibition and the resulting dNTP pool imbalances increase activity of the salvage pathway, particularly of thymidine kinase (TK) facilitating FUdR metabolic activation to FdUMP, the active metabolite of 5-FU and FUdR (see Fig. 1 for a schematic representation). A TK positive-feedback loop has been recognized as a major biochemical response after RNR inhibition mediated by hydroxyurea [34,35] and by tezacitabine itself [13]. Increased TK activity is believed to be the result of a major drop in dATP levels after RNR inhibition, as observed in different cell lines after tezacitabine treatment [11]. In addition to TK upregulation, RNR inhibition also depletes intracellular pools of deoxyuridine monophosphate and therefore enhances the binding of FdUMP to TS [3,36,37].

To evaluate the role played by the enzyme thymidine phosphorylase (TP) in HCT 116 colon cancer xenografts and in the interaction of tezacitabine and fluoropyrimidines, we tested the combination of tezacitabine and capecitabine (*N*4-pentyloxycarbonyl-5′-deoxy-5-fluorocytidine, Xeloda®), an orally administered fluoropyrimidine that is converted to 5-FU by TP [21]. This combination produced a statistically significant additivity and may be correlated to a tezacitabine-induced elevation of TP levels in HCT 116 tumor tissue. Upregulation of TP has been observed in several human cancer xenografts after treatment with a variety of agents, including the nucleoside analog gemcitabine [38]. The evidence that TP upregulation by gemcitabine resulted in a synergistic antitumor activity of gemcitabine and capecitabine prompted the evaluation of this combination in clinical trials in patients with advanced cancer [39] and showed promising results in patients with advanced pancreatic carcinoma [40]. More recently, TP expression levels in patients with colorectal cancer have been correlated with the response to capecitabine plus irinotecan therapy [41] supporting the hypothesis that TP expression is predictive of the outcome to fluoropyrimidine chemotherapy.

Recent clinical studies [32] support clinical activity of tezacitabine in combination with 5-FU in gastrointestinal tumors, including esophageal, colorectal, biliary, and gastric tumors. The relative resistance of tezacitabine to deamination by cytidine deaminase, an enzyme expressed at high levels in human malignancies of gastrointestinal origin, might explain this finding. Our results support the evidence of a significant tezacitabine-mediated modulation of the DNA-directed effects of fluoropyrimidines in human colon cancer cells. These findings suggest that a better understanding of the complex mechanism of action of fluoropyrimidines may improve the use of tezacitabine to modulate the antitumor activity of this class of drugs.

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